REGULATION OF CLONED CARDIAC CHANNELS

A Thesis

by

BHARATHI BALASUBRAMANIAN

Submitted to the Office of Graduate Studies of
Texas A&M University
in partial fulfillment of the requirements for the degree of

MASTER OF SCIENCE

August 2005

Major Subject: Biomedical Engineering

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Approved by:

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ABSTRACT

Regulation of Cloned Cardiac Channels. (August 2005)

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Activation of $a_5ß_1$ integrin potentiates L-type calcium current in vascular smooth muscle, which is partly mediated by tyrosine phoshorylation of the a1c channel subunit. Expressed rabbit VSM and neuronal isoforms are also potentiated by $a_5ß_1$ integrin activation and require dual phosphorylation of a1c by PKA and c-Src. To explore common mechanisms of regulation by $a_5ß_1$ integrin, whole cell patch clamp experiments were used to investigate the effects of $a_5ß_1$ integrin antibody on expressed cardiac calcium channels. In HEK cells transfected with a1c, ß2a and a2-d1 subunits alone, currents increased 1.8 – 2.0 fold on application of $a_5ß_1$ antibody. The potentiation was almost completely abolished on the application of PKI, a highly specific Protein Kinase A (PKA) inhibitor. The expressed currents increased 2.0 – 2.2 fold on application of PKA activator 8-Br-cAMP, and abolished by PKI. Our results suggest that regulation of L-type calcium channels by $a_5ß_1$ integrin is a general mechanism shared by VSM, neuronal and cardiac channels. However, in the cardiac isoform, only PKA phosphorylation is involved.

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CHAPTER I

INTRODUCTION

The focus of this thesis is to test whether the cardiac L-type calcium channel is potentiated by activation of $a_5ß_1$ integrin and whether the potentiation depends on phosphorylation of a channel serine residue by Protein Kinase A (PKA) and/or a tyrosine residue by c-Src. Studies on the regulation of the smooth muscle and neuronal L-type calcium channel by $a_5ß_1$ integrin were conducted previously in our lab and their mechanism of regulation has been established. Investigating the regulation of cardiac L-type calcium currents would aid in assessing common regulatory pathways for this channel between the three muscle types, viz. cardiac, neuronal and smooth muscle, based on their structural homology and thus help in understanding how they contribute to the unique behavior of the respective muscle types.

A. BACKGROUND

Calcium channels are found in every mammalian cell. By controlling the flow of calcium ions into the cytoplasm, calcium channels can regulate a host of calcium-dependent intracellular events (1).

Voltage dependent calcium channels (VDCC) are a family of calcium channels

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This thesis follows the style and format of *The Journal of Biological Chemistry*.

found in excitable cells such as nerve and muscle. VDCC activate when the membrane is depolarized and usually require a strong depolarization for significant opening. The probability of opening is steeply voltage dependent and is delayed relative to the gating of sodium channels that are also typically found in excitable cells. Compared to sodium currents, the calcium currents thus produced are quite minimal (1). Even when maximally activated, whole-cell VDCC current (I_{Ca}) rarely exceeds 100 $\mu A/cm^2$. Since I_{Ca} is generally small but slowly decaying, pure Ca^{2+} action potentials have a low rate of rise, a low conduction velocity, and a long duration (1).

Based on physiological and pharmacological criteria there are multiple types of VDCC present in various cell types, viz. P, Q, R, N, T and L. P and Q type calcium channels are found in nerve terminals and dendrites; their primary functions are neurotransmitter release and producing dendritic Ca^{2+} transients. R-type channels are located in cell bodies, dendrites and nerve terminals and are primarily responsible for generating Ca^{2+} dependent action potentials. T-type channels are found in cardiac muscle, skeletal muscle and neurons and are thought to be involved in pacemaker activity. The main focus of this thesis is on the L-type calcium channels which are located in skeletal muscle, cardiac muscle, endocrine cells, neurons and smooth muscle. L-type currents are distinguished by high voltage of activation, a relatively large single-channel conductance, slow voltage-dependent inactivation, marked regulation by intracellular kinases and specific inhibition by Ca^{2+} antagonist drugs including dihydropyridines, phenylalkylamines and benzodiazepines. Verapamil, D-600 and nifedipine are commonly used blockers of L-type calcium current (1).

In a resting mammalian cell, the cytoplasmic calcium level is held very low, typically within the range 10 – 200 nM (1). Whenever a calcium channel opens, Ca^{2+} ions enter the cytoplasm, raising the intracellular Ca^{2+} concentration transiently until buffering or pumping mechanisms tie up or remove the extra Ca^{2+}. An increase in intracellular calcium secondary to Ca^{2+} entry results in the complexing of Ca^{2+} with calmodulin (CaM) and other Ca^{2+} binding proteins. In muscle, Ca^{2+} – CaM activates Ca^{2+} – dependent regulatory contractile proteins, for example troponin in striated muscle or myosin light chain kinase in smooth muscle. The result is shortening of the contractile apparatus and development of active tension. Secretion from nerve terminals and from different types of excitable cells requires extracellular Ca^{2+}. Katz and Miledi have proposed that the opening of voltage-gated calcium channels in the presynaptic potential allow a burst of calcium ions into the cell (1). These calcium ions then bind with intracellular calcium sensors that act in unison to initiate vesicle fusion and cause exocytosis of neurotransmitter. Intracellular calcium ions also have an important role to play in gating of channels and such modulation has been reported in several Ca^{2+}, K^{+} and Cl^{-} channels. These channels are known to have intracellular binding sites for calcium or calmodulin and are known to have a direct effect on gating (1).

Based on their tissue-specific expression, L-type calcium channels can be classified as neuronal, smooth muscle, skeletal or cardiac channels. Neuronal, smooth muscle and cardiac channels are of one class (α_{1C}); skeletal muscle channels are of a separate class (α_{1D}). The main emphasis of this thesis is on the cardiac calcium channel subclass, $\alpha_{1C\text{-}a}$. In all four tissues, L-type channels are regulated by a number of

hormones, neurotransmitters and intracellular kinases, thereby allowing the cells to have exquisite control of extracellular calcium entry. The intracellular kinases whose role in the regulation of L – type channels are of primary interest in this thesis are Protein Kinase A (PKA) and pp^{60}c-Src.

In describing the rationale for studying the regulation of L – type currents by these kinases, a discussion of the molecular structure and properties of Ca^{2+} channels is appropriate. The primary structure of the Ca^{2+} channel (Fig 1) consists of the pore-forming a_1 subunit, composed of amino acid sequences organized in four repeating domains (I to IV), each of which contains six transmembrane segments (S1 to S6) and an intracellular, membrane-associated loop between transmembrane segments S5 and S6 (2). The S4 segment of the

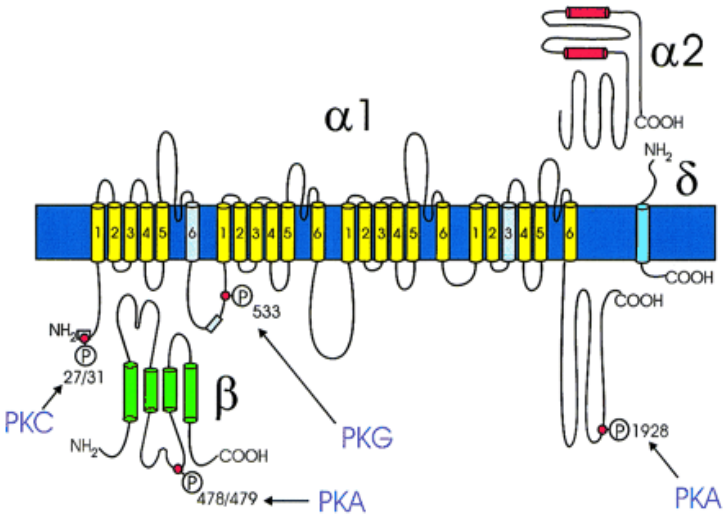

Fig 1. Structure of L-type calcium channel (Keef et al., Am J Physiol 281:C1743-C1756, 2001).

a_1 subunit serves as the voltage sensor for activation. The S5 and S6 segments and the 5-6 loop constitute the pore. S6 is the receptor site for L-type specific Ca^{2+} antagonists. An intracellular ß - subunit contains a domain that interacts with the I – II loop of the a_1 subunit. The functions of the ß subunit are plasma membrane targeting and modulation of channel gating. The channel also consists of a d subunit, which is a membrane protein and an a_2 subunit, which is an extracellular glycosylated protein. The roles of the a_2 – d subunits are less understood but both subunits have been identified to play a role in the modulation of gating and membrane targeting (3).

B. REGULATION BY PKA

Protein Kinase A is widely expressed in cardiac and smooth muscle (4). The basic structure of PKA consists of two catalytic subunits bound to a regulatory subunit. A large number of G-protein coupled receptors in the heart and blood vessels are known to regulate the L-type calcium channel and other cellular proteins through the cAMP/PKA pathway (5). These receptors are coupled to either G_s, which stimulates, or G_i, which inhibits adenyl cyclase (AC). An increase in AC activity produces an increase in cellular cAMP which in turn binds to cAMP-dependent regulatory PKA subunits leading to phosphorylation of substrates on specific serine and/or threonine residues (6). Evidence for the regulation of L type calcium channels by PKA has been provided by electrophysiological studies performed in multiple laboratories. Agonists of ß Adrenergic Receptor (ßAR) signaling, predominantly $ß_1$AR, produce an increase in I_{Ca} involving the cAMP/PKA pathway (3). In cardiac muscle, PKA phosphorylates a

number of intracellular proteins, promoting enhanced speed of contraction and relaxations.

Evidence for direct phosphorylation of L-type channels by PKA has come from biochemical studies. Experiments where the full-length form of a_{1C} was truncated at its C-terminus showed that only the full-length form was phosphorylated by PKA; thus, the PKA phosphorylation site was situated in the truncated portion. Serine 1928 in the α_{1C} C-terminus has been identified as the phosphorylation site (6). These results has been further supported by experiments where mutation of Serine 1928 to Alanine, prevented phosphorylation of channel by PKA (5). It has also been shown that the β calcium channel subunit is responsible for a smaller fraction of current potentiation in the same cells (7).

C. REGULATION BY PKC

Multiple G_q protein-coupled receptors including endothelin (ET), a1 adrenergic and angiotensin II receptors, trigger events leading to the activation of PKC (6). The regulation of L-type calcium channels by PKC is controversial. Direct activators of PKC produce biphasic effects on I_{Ca} in certain preparations. These diverse effects may be due in part to the existence of at least 11 different isoforms of PKC which are divided into three groups:

1) classic or conventional PKCs (cPKC) that are activated by diacyl glycerol and are calcium sensitive;

2) novel or new PKCs (nPKC) that are activated by diacylglycerol or phorbol ester but are calcium insensitive;

3) atypical PKCs (aPKC) that are not activated by diacylglycerol or phorbol ester and are calcium insensitive (6).

In the cardiac muscle cell, PKC phosphorylates a number of proteins which contribute to excitation and contraction. Amongst these is the L-type calcium channel in which both the a1 and the ß2 subunits are phosphorylated by PKC in vitro. Experimental studies have shown that the first 46 amino acids at the NH_2 terminal end of the cardiac a1 subunit inhibit channel activity (8, 5). Removal of these residues results in enhancement of channel activity by PKC. Also, there is evidence that different isoforms of PKC have different effects, with cPKCs producing inhibition of current and nPKCs enhancing current. Further studies show that for channels expressed in TSA-201 cells, inhibition of channel activity by PKC is through phosphorylation of Threonine 27 and Threonine 31 at the NH_2 terminus end of the a1 subunit (9).

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D. REGULATION BY PKG

PKG catalyzes the phosphorylation of a number of intracellular proteins that modulate muscle contraction. There are two main types of PKG, Type I and Type II. The cardiac muscle cell contains PKG Type I (9). There is controversy in the role of the cGMP/PKG pathway in regulation of myocardial function, with some studies reporting cGMP/PKG mediated inhibition of L-type calcium channels, while others report the opposite effect especially when the cAMP levels are high. But in most cases where

potentiation was observed, the mechanism was not through the direct phosphorylation of PKG, but was indirect through PKA activation. Three different mechanisms have been suggested for cGMP-induced inhibition of L-type calcium channels, one of which is through the direct phosphorylation of the channel by PKG on Serine 533 (10).

The previous discussion was based on results obtained from experiments that investigated each pathway individually without the contribution of other pathways. In real physiological systems, the channel is regulated by a complex mixture of cellular signals and a thorough understanding of the various interactions between signaling cascades is absolutely necessary to obtain a complete picture of how the channel is regulated.

E. REGULATION OF CALCIUM CHANNELS BY TYROSINE KINASES

Protein Tyrosine Kinases (PTK) were discovered as products of oncogenes from transforming retroviruses and are responsible for transducing key extracellular signals that mediate events such as proliferation, cytoskeletal rearrangement and coordination of physiological responses (11). PTKs are of two types, receptor and non-receptor tyrosine kinases. Several studies have shown that PTKs regulate VDCC, including pp^{60}c-Src (11). In smooth muscle myocytes, PTK inhibitors produce a reduction in Ca^{2+} current, whereas protein tyrosine phosphatases (PTP) inhibition increases current (12). The intracellular application of constitutively active Src kinases increases $I_{Ca.}$ c-Src-activating peptide also has a similar effect, whereas a monoclonal antibody for c-Src inhibits I_{Ca} (12, 13).

G. INTEGRINS-FUNCTIONS AND PHYSIOLOGICAL RELEVANCE

Integrins are heterodimeric transmembrane proteins that play a very important functional role in connecting the extracellular matrix (ECM) to the cell body or the cytoskeleton (12). Integrins are also known to regulate the L-type calcium channel. Each integrin is composed of a single a and ß subunit. Different combinations of 18a and 8 ß subunits result in the formation of more than 24 integrin family dimers (13). The ligand specificity of the integrin is determined by the combination of a and ß subunits, but many of these tend to overlap. Under these circumstances, the amount of integrin expression/activation pattern and the availability of the ligand specifies functional interactions taking place in vivo. The integrin of interest in this thesis is the $a_5ß_1$ integrin. The ECM ligand for this integrin is fibronectin (14).

Physiologically, integrins play an important role in cell-cell adhesion, cell-ECM adhesion, wound healing, cell proliferation, differentiation, migration and death (15). Integrins are particularly important in cardiac development (16). Integrins are the most important receptors in the ECM and hence their appropriate expression and functioning is indispensable for normal cardiac activity (17, 18). They are also known to play an important role in apotosis, by influencing signaling pathways and thereby either causing survival or death of the cell by its interaction with the ECM (19). The $a_5ß_1$ integrin, along with its ECM ligand fibronectin, also helps in wound healing (20).

F. MECHANISMS OF INTEGRIN REGULATION

Recent studies (21, 23) show that the L-type calcium channels in neurons and blood vessels are regulated by the $a_5ß_1$ integrin through serine and tyrosine dependent pathways. Activation of a5ß1 integrin increases L-type calcium currents (21). In vascular smooth muscle, integrin-mediated regulation of the L-type channel occurs through multiple intracellular pathways. The inhibition of c-Src or its upstream, integrin-associated partner Focal Adhesion Kinase (FAK) with intracellular antibodies inhibits potentiation of current following $a_5ß_1$ integrin activation (21). Application of antibodies to two integrin associated protein targets, paxillin and vinculin, also block regulation by $a_5ß_1$. The latter effect may be due to the role of these proteins in acting as a scaffold on which various regulatory proteins associate with PTKs. The $a_5ß_1$ integrin thus seems to regulate the protein tyrosine phosphorylation cascade involving c-Src and FAK.

Platelet-derived-growth-factor (PDGF) and other growth factors increase I_{Ca} by stimulating tyrosine phosphorylation of multiple proteins. PDGF regulation of current is inhibited by antibodies to FAK (24) and c-Src (24, 25). Insulin Growth Factor (IGF-I) is also known to regulate L-type current in neurons through c-Src (22).

Recently, Gui et al. (23) have shown that recombinant neuronal and smooth muscle cells are acutely regulated by $a_5ß_1$ integrin through a pathway primarily involving the a_{1C} channel subunit. Further studies have shown that this regulation of rat neuronal/ vascular smooth muscle channels by $a_5ß_1$ integrin involves dual-phosphorylation of C-terminal residues S1901 and Y2122 (using the neuronal calcium channel numbering scheme) which undergo direct phosphorylation in the presence PKA

and c-Src respectively (23). The mechanism is supported by experiments performed in native smooth muscle and neuronal cells in which potentiation by a5ß1 integrin was blocked fully by a combination of PKA inhibitor and c-Src SH2 inhibitory peptide (23).

G. a1c C-TERMINUS STRUCTURAL HOMOLOGY

There is structural homology in the C-terminus of the a1c subunit in the rat neuronal calcium channel (studied by Gui et al. (23)) and the rabbit cardiac channel which is the channel of interest for my experiments. S1901 (the PKA phosphorylation site), which has been identified as the site responsible for potentiation of the channel by a5ß1 integrin in rat neuronal cells, corresponds to S1928 in the rabbit cardiac channel C-terminus (3). Notably, the rabbit cardiac channel lacks the critical tyrosine residue (corresponding to Y2122 on rat neuronal calcium channel) which has been found to mediate ~50% of potentiation by a5ß1 integrin in the neuronal and smooth muscle isoforms (3). Considering this structural homology, a primary goal of this thesis is to test whether the cardiac L-type calcium channel is also potentiated by $a_5ß_1$ integrin activation and whether the potentiation depends on a1c phosphorylation by PKA on S1928 and/or a tyrosine residue by c-Src.

CHAPTER II

EXPERIMENTAL MATERIALS AND METHODS

A. PATCH CLAMP TECHNIQUE

The primary technique employed in this thesis is Patch Clamping, which is a widely used method to study ionic currents in single cells (26). The essence of patch clamp recording is the use of a fire-polished glass micropipette and suction to form a high resistance seal between the pipette and cell membrane, combined with a low-noise current-to-voltage converter amplifier. The method can be used to record single channels or a population of channels, the so-called Whole-Cell Recording (WCR), which is the most common configuration of the patch clamp technique.

Whole Cell Recording

WCR enables the experimenter to manipulate the internal environment of the cell by using a patch pipette filled with appropriate solution. The pipette is pressed onto the cell membrane to establish a firm seal with a resistance in the order of gigaohms. The pipette voltage is set at a negative voltage to mimic the normal transmembrane electrical gradient and positive voltage steps of few millivolts are applied to gate VDCCs. Appropriate compensation techniques are employed in order to cancel the transients caused by the capacitance between the holder and the pipette wall. The cell membrane is then ruptured by applying gentle suction (Fig. 2), after which the pipette solution

exchanges with the intracellular solution. After complete exchange of the intracellular solution, the currents obtained are amplified and recorded.

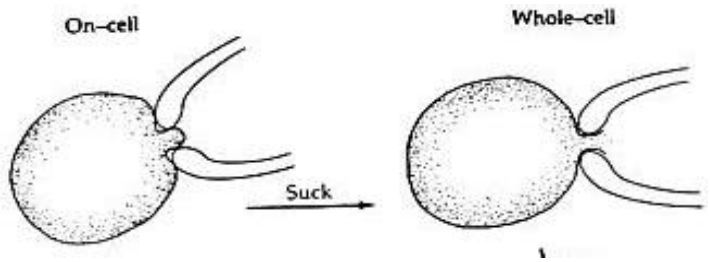

Fig 2. Whole-Cell patch clamp configuration (http://www.bphys.uni-linz.ac.at/bioph/res/icg/met/patch.htm).

Fabrication of Patch Pipettes

The pipettes used in patch clamping play a very important role in the formation of a gigaseal. The glass geometry and the pipette tip shape determine the quality of the recording and the stability of the seal. A lot of attention is paid to these factors to achieve a good seal and stable recording. The pipette glass is chosen according to the type of the cell being patched. For the proposed set of experiments involving HEK 293 cells with WCR, 8161 glass was chosen for the recording pipette. The glass was then cleaned and pulled using a Sutter PC-97 horizontal pipette puller. To promote the formation of a gigaseal and to prevent pipette tip penetration into the cell, the pipettes were fire-polished. Fire-polishing was accomplished by heating up a short platinum wire by passing D.C. current through it. The pipette was brought close to the heated wire while observing it under a microscope and was polished until the tip was slightly

rounded. Care was taken to see that the pipette tip was not blocked completely by polishing. The fire-polished tips had diameters of 1 – 3 µm and resistances of 3 – 5M? .

A second pipette, called the picospritzer pipette (connected to a Parker-Hannefin picospritzer), was used to puff small volumes of integrin antibody or 8-Br-cAMP onto the cell from a distance of ~5 µm. 7052 glass was chosen for this purpose and was cleaned and pulled using the horizontal pipette puller. The picospritzer pipette was not firepolished since it did not have direct contact with the cell membrane.

B. CELL CULTURE AND TRANSFECTION

HEK 293 cells transfected with the rabbit cardiac channel were used for these experiments. HEK cells were used rather than native cardiac myocytes to enable channel mutations to be performed and their effects tested. HEK cells were a convenient cell line to use because they do not express endogenous VDCCs but do express $a_5ß_1$ integrin.

HEK cells were plated at 60% density one day before transfection in 10% FBS DMEM. 1-1.5 ml cell suspension was diluted into 4ml fresh 10% FBS DMEM. When the cells were 80% confluent, transfection was performed using the lipofection technique in 60mm dishes. 20µL of Lipofectamine 2000 and 14µg of the plasmid cDNA encoding the rabbit cardiac L-type calcium channel (7µg a1c, 3.5 µg ß2a, 3.5 µg a2-d1) were added to the dish and the dish was placed in a 37ºC incubator with 5% CO_2 for 20 hours. Green fluorescent Protein (GFP) 2µg/dish was also added to allow identification of transfected cells. The next day, the cells were split with 0.05% trypsin and 0.53mM

EDTA and plated on sterile coverslips in 35mM dishes. Cells were maintained at 28ºC with 5% CO_2 for 20 hours.

C. SOLUTIONS

The calcium currents were recorded using the whole cell method with bath solution containing in (mmol/L) 20 $BaCl_2$, 124 Choline chloride, 10 Hepes and 15-D glucose. The pH was adjusted to 7.4 using TEA-OH. Barium was used in the bath solution in order to obtain sustained calcium currents and block potassium currents. The pipette solution contained (mmol/L): CsCl 104.5, TEA-Cl 20, EGTA 11, $MgCl_2$ 2, $CaCl_2$ 1, Hepes 10 and GTP 0.2. The pH was adjusted to 7.2 with CsOH. Cs^+ was used in order to further block potassium currents.

D. EXPERIMENTAL SETUP

A coverslip containing the transfected HEK 293 cells was placed in the recording chamber. Bath solution was perfused through the recording chamber at a flow rate of 1-3mL/min from a switchable, gravity-fed reservoir system. The cells were observed under a Zeiss microscope and fluorescent cells (those expressing GFP) were chosen for patch clamping. The recording pipette was backfilled with pipette solution in such a way as to eliminate air bubble formation at the pipette tip, clamped into the pipette holder, and connected to the preamplifier headstage. The pipette was pressed against the membrane to form a gigaseal, and then suction was applied to break open the membrane. The holding potential was -80mV to keep the channels inactivated. Intracellular and

extracellular solutions were allowed to exchange for approximately ten minutes to attain equilibrium. The currents were then measured using ramp (-100 to +80 mV for 180 msec) and step voltage protocols (a series of steps from -80 to +80 mV for 300 msec duration at 5 second intervals). The currents were amplified using a HEKA EPC9 amplifier under the control of Pulse Software. The resolution of currents was ~1pA.

CHAPTER III

EXPERIMENTAL PROTOCOLS AND RESULTS

The following experiments were designed to investigate the mechanism of cardiac L-type calcium channel regulation by $a_5ß_1$ integrin activation. Whole-cell patch clamp recordings were performed on HEK 293 cells transfected with a_{1C}, $ß_{2a}$ and a_2-d_1 subunits of the rabbit cardiac calcium channel.

A. CARDIAC a1c POTENTIATION BY $a_5ß_1$ INTEGRIN ANTIBODY (Ab)

This protocol was designed to test if $a_5ß_1$ integrin activation results in potentiation of the cardiac L-type calcium channel and, if so, to determine the amount of potentiation. $a_5ß_1$ integrin is constitutively expressed by HEK cells. To activate the integrin, soluble $a_5ß_1$ integrin Ab (10µg/L) was applied to the cell through a picospritzer micropipette for 5 minutes. At this concentration, the Ab is known to cross-link and activate the integrin. The cell was held initially at -80 mV (Fig. 3) because at this potential most of the calcium channels are closed. The membrane potential was then stepped to 10 mV (Fig. 3) where voltage peak inward current is recorded. This procedure was repeated continuously for up to 20 min, allowing 10-20 between voltage steps fro recovery of current. During this time, application of $a_5ß_1$ integrin Ab produced potentiation of whole-cell calcium current. The average potentiation in the absence of inhibitors was ~91% above the basal current level (9 cells). The raw traces of whole cell current before (Control) and after application of integrin are shown in Fig. 3.

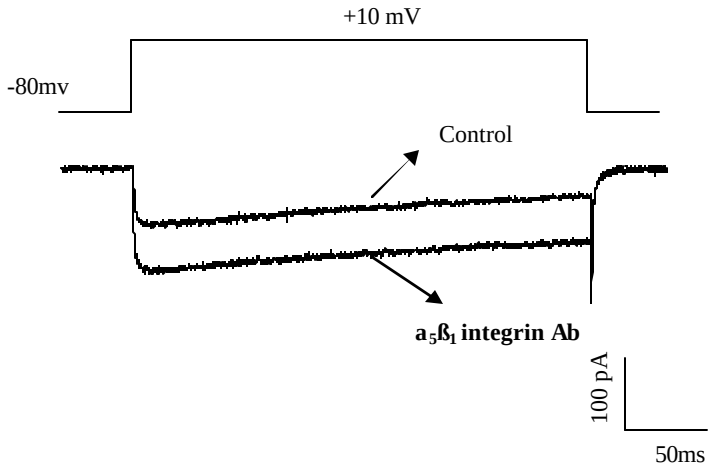

Fig 3. Potentiation of cardiac L-type calcium channel currents by $a_5ß_1$ integrin. Raw current traces showing effect of $a_5ß_1$ integrin Ab (10 µg/L) on calcium current in HEK 293 cells transfected with a_{1C}, $ß_{2a}$ and a_2-d_1 subunits of rabbit cardiac channels.

Table 1 shows the measured control currents and potentiated currents after $a_5ß_1$ integrin activation in 9 cells expressing cardiac L-type calcium channels. The percentage increase in potentiation was measured in this protocol and all subsequent protocols by dividing the difference between the control and the potentiated current by the control current and multiplying the quotient by 100. Thus, each cell was used as its own control, which minimizes the variability associated with substantially different channel expression levels from cell to cell (Table 1, first column). The average potentiation for

all the 9 cells was found to be 91.1 % with a standard error of 9.5%. To test significant differences between the control and potentiated groups, a paired t-test with a significance level of 0.05 was used. The p-value was found to be 0.0004. Since $p<0.05$, it was concluded that there is significant potentiation produced by application of $a_5ß_1$ integrin. In cells not transfected with L-type Ca^{2+} channels, no voltage-gated Ca^{2+} currents were detected and $\alpha_5\beta_1$ integrin activation produced no effect.

Table 1. Summary data for potentiation of cardiac L-type calcium channel currents by $a_5ß_1$ integrin.

Control (pA)	a5ß1 integrin activation (Potentiated) (pA)	% Increase in Potentiation above control currents
136.3	274	101.0
658	1276	93.9
170	325	91.2
185	360	94.6
338	679	100.9
545	1098	101.5
1206	1437	19.2
338	750	121.9
358	700	95.5

n = 9	
Average % Increase in Potentiation	**91.1**
Standard Error	**9.5**
Paired t-test comparing control currents with a5ß1 integrin (Potentiated) currents	**0.0005**
Significance level	**a=0.05**

B. CARDIAC a1c POTENTIATION BY 8-Br-cAMP

8-Br-cAMP is direct, cell-permeable PKA activator known to potentiate L-type calcium currents through the PKA pathway as discussed in Chapter I. Given the previous

work by our lab that PKA is involved in potentiation of the neuronal calcium channel following $a_5ß_1$ integrin activation, this protocol was performed to compare the cardiac a1c potentiation by $a_5ß_1$ integrin antibody with potentiation by 8-Br-cAMP. We hypothesized there would similar amounts of potentiation produced by the two compounds if integrin-mediated potentiation were mediated solely through the PKA pathway.

8-Br-cAMP (1 mM) was applied to the transfected HEK 293 cells for 5 minutes through a picospritzer micropipette. An increase in calcium current was observed within 1 min upon application of 8-Br-cAMP. The average potentiation from 11 cells was ~123%. Sample raw traces of whole cell control and potentiated currents are shown in Fig 4.

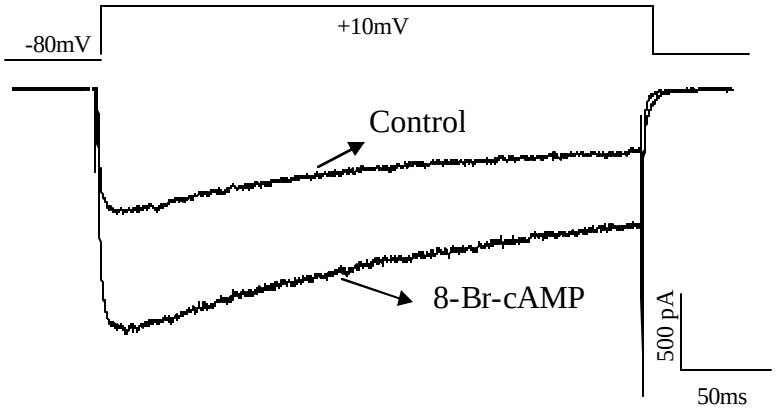

Fig. 4. Potentiation of cardiac L-type calcium channels by application of the PKA activator 8-Br-cAMP. Raw current traces showing effect of 8-Br-cAMP (1 mM) in HEK 293 cells transfected with a_{1C}, $ß_{2a}$ and a_2-d_1 subunits of the rabbit cardiac channel.

Table 2 shows the measured control currents and potentiated currents produced by 8-Br-cAMP in 11 cells expressing cardiac L-type calcium channels. The average potentiation was found to be 123% with a standard error of 14.3%. To evaluate the significance of differences between the control and potentiated groups, a paired t-test with a significance level of 0.05 was used. The p-value was found to be 0.0006. Since $p<0.05$, it was concluded that significant potentiation was produced by application of 8-Br-cAMP.

Table 2. Summary data of potentiation of cardiac L-type calcium channel currents by 8-Br-cAMP.

Control (pA)	8BrcAMP Potentiation (pA)	% Increase in Potentiation above control currents
173.4	231.2	33.3
136	347	155.1
173	390	125.4
668	850	27.2
115	284	147.0
99	230	132.3
361	809	124.1
173	425	145.7
273	710	160.1
180	454	152.2
120	300	150.0

n = 11	
Average % Increase in Potentiation	**123.0**
Standard Error	**14.3**
Two tailed t-tests comparing Control and 8-Br-cAMP Potentiated currents (pA)	**p = 0.0006**
Significance level	**a = 0.05**

C. EFFECT OF PKI IN THE RECORDING PIPETTE DURING $a_5ß_1$ INTEGRIN Ab-MEDIATED POTENTIATION OF CURRENT

This protocol was performed to test if the potentiation produced by $a_5ß_1$ integrin could be prevented by PKI, a highly selective inhibitor of PKA. The results from this experiment would show if the potentiation produced by $a_5ß_1$ integrin was achieved completely through the PKA pathway.

PKI is a peptide and is not cell-permeable, unlike 8-Br-cAMP, and so it could not be applied to the cell through a picospritzer micropipette. Therefore PKI (5µg/ml) was mixed with the pipette solution and filled in the recording pipette.

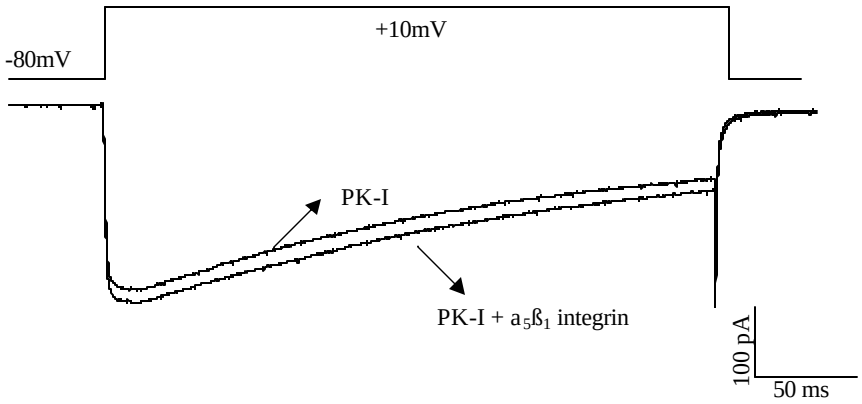

Fig. 5. Effect of the PKA phosphorylation inhibitor PKI on potentiation of cardiac L-type calcium channel currents by $a_5ß_1$ integrin. Raw current traces showing effect of PKI (5µg/ml) in the recording pipette on $a_5ß_1$ integrin potentiation in HEK 293 cells transfected with a_{1C}, $ß_{2a}$ and a_2-d_1 subunits of the rabbit cardiac channels. The small blips in this recording are 60 Hz noise.

Control currents (PKI alone) were measured and recorded before the application of $a_5ß_1$ integrin antibody. The integrin antibody (10 µg/ml) was then applied to the cell through a picospritzer for 5 minutes while delivering test pulses from -80 mV to +30 mV and recording current. Under these conditions, the potentiation produced by $a_5ß_1$ integrin was only a fraction of that recorded in the absence of inhibitor. The average potentiation was ~6% in 7 cells. Sample recordings of control and potentiated currents are shown in Fig 5.

Table 3 shows the peak Ca2+ currents for cells dialyzed with PKI alone and for cells dialyzed with PKI and then exposed to $a_5ß_1$ integrin AB. The average potentiation for 7 cells was found to be 5.9% with a standard error of 1.4%.

Table 3. Summary data for effect of PKI on $a_5ß_1$ integrin potentiation of cardiac L-type calcium channel currents.

PKI (pA)	PKI + a5ß1 integrin Ab (pA)	% Increase in potentiation above basal currents
174	193	9.8
153	169	10.5
340	343	0.9
190	201	5.8
410	423	3.2
144	155	7.6
670	689	2.8
	n=7	
	Average % Increase in potentiation	**5.9**
	Standard Error	**1.4**
	Paired t-test comparing control (PK-I) and potentiated currents (PK-I + a5ß1 integrin)	**0.0008**

Paired t-tests were performed between the PKI and PKI+$a_5ß_1$ integrin Ab groups and no significant potentiation to $a_5ß_1$ integrin activation was found in the presence of PKI.

To further test if PKI blocked the potentiation of current by $a_5ß_1$ integrin activation, we also evaluated the significance of differences between currents potentiated by$a_5ß_1$ integrin Ab (Table 1) and currents potentiated by a5ß1 integrin in the presence of PKI (Table 3). The approach used is different from the one used in Chapter III, Sections A and B for evaluation of significant differences. In the present case we compared currents between two different groups, therefore a two-tailed t-test with unequal sample variance was performed.

Table 4. Evaluation of significance of differences between $a_5ß_1$ integrin activated currents (control) and PKI + $a_5ß_1$ integrin (potentiated currents).

a5ß1 integrin-Ab Potentiated currents (% increase above control current)	PKI + a5ß1 integrin-Ab (pA)
101.0	9.8
93.9	10.5
91.2	0.9
94.6	5.8
100.9	3.2
101.5	7.6
19.2	2.8
121.9	
95.5	

p-value comparing a5ß1 integrin potentiated currents and currents (Control) and PKA I + a5b1 Integrin (Potentiated) currents	**p= 0.0001**
Significance level	**a=0.05**

The results are tabulated in Table 4. The value of p was found to be 0.0001. Since $p<0.05$, it was concluded that there was a significant difference between the two groups, showing that the addition of PKI blocked potentiation of current produced by a5ß1 integrin activation.

D. EFFECT OF PKI IN RECORDING PIPETTE ON 8-Br-cAMP POTENTIATION

The final protocol was designed to test whether 8-Br-cAMP potentiation was

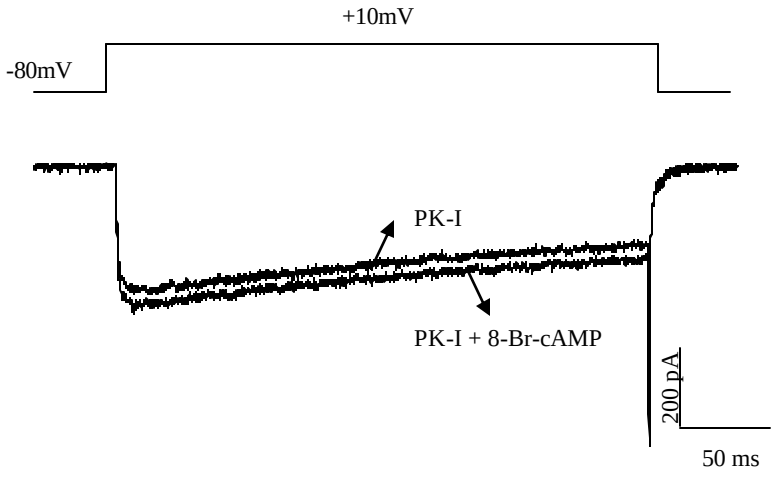

Fig. 6. Effect of the PKA phosphorylation inhibitor PKI on potentiation of cardiac L-type calcium channel currents by 8-Br-cAMP. Raw current traces showing the effect of PKI (5 µg/ml) in recording pipette on 8-Br-cAMP potentiation in HEK 293 cells transfected with a_{1C}, $ß_{2a}$ and a_2-d_1 subunits of the rabbit cardiac channel.

blocked by PKI in the recording pipette. If 8-Br-cAMP effects were inhibited solely by PKA, the potentiation should be completely blocked by PKI.

PK-I (5 µg/ml) was added to the pipette solution. Control and potentiated currents were measured before and after the application of 8-Br-cAMP (1 mM). Raw traces of control and potentiated currents are shown in Fig. 6.

Table 5. Summary data for effect of PKI on 8-Br-cAMP potentiation of expressed cardiac L-type calcium channel currents.

PK I (Control) (pA)	PKI +8-Br-cAMP (pA)	% Increase in Potentiation above control current
139	150	7.9
89	93	4.5
169	180	6.5
115	127	10.4
230	248	7.8
520	538	3.5
236	252	6.8
	n=7	
	Average % Increase in Potentiation	**6.8**
	Standard Error	**0.9**
	t-tests comparing control and potentiated groups	**p=0.0004**

The average potentiation to 8-Br-cAMP in the presence of PKI was 6.7% in 7 cells. Paired t-tests were performed between the PKI and PKI+8-Br-cAMP groups and it was found that no significant potentiation wa produced by 8-Br-cAMP in the presence of PKI. The data for 7 cells are tabulated in Table 5. The average potentiation in the presence of PKI was found to be 6.7% with a standard error of 0.9%. To further compare 8-Br-cAMP effects in the presence and absence of PKI, (8-Br-cAMP activated currents

from Table 2 and currents for PKI + 8-Br-cAMP from Table 5 were statistically tested. The comparisons are shown in Table 6.

Table 6. Evaluation of significance of differences between 8-Br-cAMP activated currents (control) and PKI + 8-Br-cAMP (potentiated currents).

8BrcAMP activation (% increase above control)	PKI +8BrcAMP activation (% increase above control)
33.3	7.9
155.1	4.5
125.4	6.5
27.2	10.4
147.0	7.8
132.3	3.5
124.1	6.8
145.7	
160.1	
152.2	
150.0	
Average potentiation	**6.8**
Standard error	**2.3**
p-value comparing 8-Br-cAMP activated currents (Control) and PKI + 8-Br-cAMP	**p = 0.00006**

A two-tailed t-test with sample unequal variances was performed. The p value was found to be 0.00006. Since $p<0.05$, it was concluded that there is significant difference effect of PKI significantly attenuated the effects of 8-Br-cAMP. This result verifies that the potentiation produced by 8-Br-cAMP was mediated solely through PKA.

CHAPTER IV

SUMMARY AND CONCLUSIONS

The data obtained from the experiments on potentiation of cardiac Ca^{2+} channel current by $a_5ß_1$ integrin Ab in the presence of the PKA inhibitor, PKI, are summarized in Fig 7.

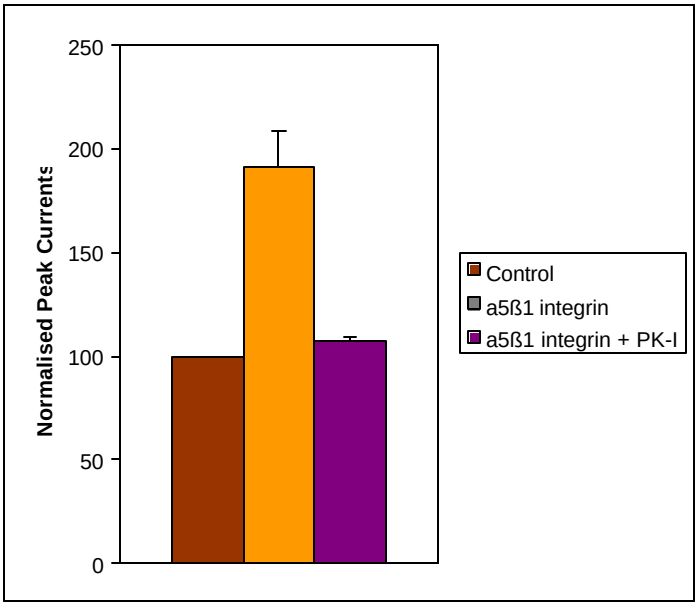

Fig 7. $a_5ß_1$ integrin + PKI summary data. Summary data (peak currents) for HEK 293 cells transfected with a_{1C}, $ß_{2a}$ and a_2-d_1 subunits of the rabbit cardiac channel after application of $a_5ß_1$ integrin Ab alone (n=9) or $a_5ß_1$ integrin Ab during dialysis with PKI (n = 7).

The data obtained from the experiments testing potentiation of expressed cardiac channels by 8-Br-cAMP and the effect of PKI inhibitor on potentiation are summarized in Fig 8.

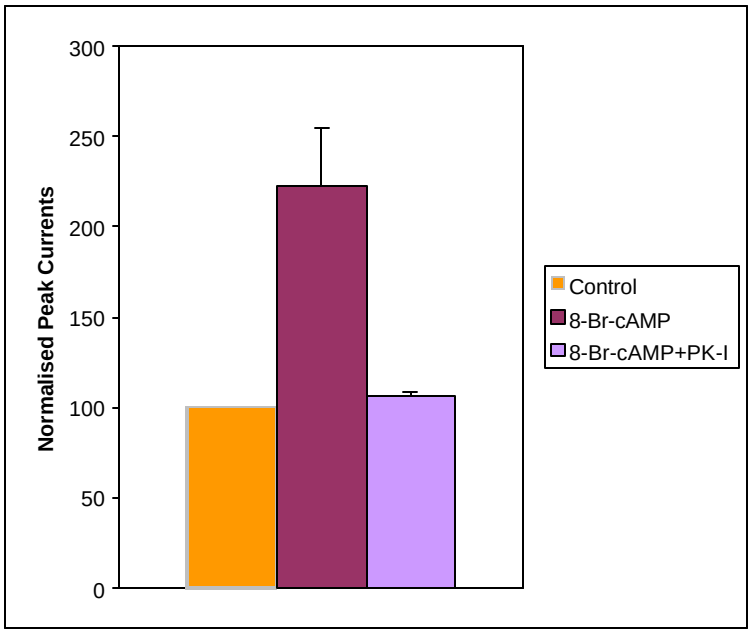

Fig 8. 8-Br-cAMP + PKI summary data. Summary data (peak currents) for HEK 293 cells transfected with a_{1C}, $ß_{2a}$ and a_2-d_1 subunits of the rabbit cardiac channel after application of 8-Br-cAMP alone (n=11) or 8-Br-cAMP plus dialysis with PKI (n = 7).

Summary data from Figures 7 and 8 show that the amount of potentiation produced by a5ß1 integrin activation (~190%) is roughly similar to the amount of potentiation produced by PKA activator 8-Br-cAMP activation (~220%). Furthermore, both types of potentiation were essentially (~95%) blocked in the presence of the PKA inhibitor PKI.

These findings are similar to previous work in our lab on native smooth muscle and the neuronal L-type calcium channels, showing that heterologously expressed cardiac L-type calcium channels are also potentiated by $a_5ß_1$ integrin activation. It can

therefore be concluded that the regulation of L-type calcium channels by $a_5ß_1$ integrin is a general mechanism shared by vascular smooth muscle, neuronal and cardiac channels. In the case of smooth muscle and neuronal L-type calcium channels, the mechanism of potentiation involves dual phosphorylation

```
Rat   neuronal 1845 SEEVEYCSEP SLLSTDILSY QDDENRQLTC LEEDKREIQP SPKRSFLRSA SLGRRASFHL
Rat     aortic 1846 ---A------ ------MF-- -E--H----- P---------- ---------- ------S---
Rabbit cardiac 1877 G-DA-C---- -----EM--- --------AP P--E--D-RL ---KG----- ------S---
Human  cardiac 1920 NHDT-A---- -----EM--- ---------L P-----D-RQ ----G----- ------S---
Consensus        .e#.E.CSEP SLLST#mlSY Q#DEnRQLt. pEE#KR#Ir. sPKrqFLRSA SLGRRASFHL
```

Fig 9. Sequence comparisons showing PKA phosphorylation sites. The amino acids marked in bold show the homologous Serine residues identified as responsible for direct phosphorylation of channel by PKA in various cell types. "-" indicates same sequence as rat neuronal channel.

of the channel by both PKA and c-Src (23), whereas potentiation of the cardiac calcium channel appears to be mediated entirely by PKA, presumably through channel phosphorylation at S1928. This result is consistent with the known sequence differences in the C terminus of the rat neuronal, rat smooth muscle, and rabbit cardiac channels. Fig. 9 shows sequence comparisons of the end of the a_{1C} C-terminus that have been identified as critical for channel phosphorylation by PKA in the rat neuronal and rabbit smooth muscle isoforms (23). S1928 of the rabbit cardiac channel is homologous to S1901 of the rat neuronal cell (identified as the site responsible for direct channel phosphorylation by PKA). We predict that S1928 is also involved in the direct

phosphorylation of the cardiac channel by PKA following potentiation by $a_5ß_1$ integrin, since that is the only known PKA phosphorylation site on the α_1 channel subunit.

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					accession#
Rat	neuronal	2085	ILSGGAQQSP NGTLLPFVNC RDPGQDRAV* VPEDESCV**Y**A LGRGRSEEAL PDSRSYVSNL	2144 2143	JH0427
Rat	aortic	2089	---------- --T------- ---------* A------A**Y**- ---------- A---------	2148 2169	Q01815
Rabbit	cardiac	2088	------R--- ---------R ----R---GQ NEQ-ASGA**C**- P-C-Q----- A-R-AG--S-	2148 2171	P15381
Human	cardiac	2162	------P--- --A------- --A-----G* GE--AG--**R**- R--AP---E- Q---V---S-	2221 2225	Q13936
Consensus			ILSGGA.QSP NGtLLPFVNc RDpGqDRAg .e#Dascvy**A** .G.g.SEEaL .DsR.yVSsL		

Fig. 10. Sequence comparisons showing tyrosine phosphorylation sites. The letters marked in bold show the homologous tyrosine residues identified as responsible for direct phosphorylation of channel by c-Src. "-" indicates same sequence as rat neuronal channel.

Fig. 10 compares the amino acid sequence near the end of the of a_{1C} C-terminus which has been identified as critical for c-Src induced phosphorylation of channel in rat neuronal and smooth muscle cells. Gui et al. (7) identified Y2122 (marked in bold in the rat neuronal sequence) as responsible for phosphorylation of the channel in that particular species. This site was responsible for ~50% of the potentiation produced by $a_5ß_1$ integrin. Note that this residue is missing in the rabbit cardiac sequence (replaced by C in the rabbit cardiac sequence). The alternate sequence is most likely the reason that the regulation of the rabbit cardiac channel seems to be mediated completely by PKA phosphorylation of the channel.

In conclusion, my results show that the cardiac L-type calcium channel is potentiated by $a_5ß_1$ integrin activation and that the potentiation is mediated completely

by PKA, presumably involving direct channel phosphorylation at S1928 on the C-terminus of the channels a_{1C} subunit.

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